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Molecular characterization of a new type of receptor-like kinase (*wlrk*) gene family in wheat

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Abstract

In plants, several types of receptor-like kinases (RLK) have been isolated and characterized based on the sequence of their extracellular domains. Some of these RLKs have been demonstrated to be involved in plant development or in the reaction to environmental signals. Here, we describe a RLK gene family in wheat (*wlrk*, wheat leaf rust kinase) with a new type of extracellular domain. A member of this new gene family has previously been shown to cosegregate with the leaf rust resistance gene *Lr10*. The diversity of the *wlrk* gene family was studied by cloning the extracellular domain of different members of the family. Sequence comparisons demonstrated that the extracellular domain consists of three very conserved regions interrupted by three variable regions. Linkage analysis indicated that the *wlrk* genes are specifically located on chromosome group 1 in wheat and on the corresponding chromosomes of other members of the Triticeae family. The *wlrk* genes are constitutively expressed in the aerial parts of the plant whereas no expression was detected in roots. Protein immunoblots demonstrated that the WLRK protein coded by the *Lrk10* gene is an intrinsic plasma membrane protein. This is consistent with the hypothesis that WLRK proteins are receptor protein kinases localized to the cell surface. In addition, we present preliminary evidence that other disease resistance loci in wheat contain genes which are related to *wlrk*.

Introduction

In animal cells, many signals are perceived by transmembrane receptors and transduced by activation of intrinsic protein kinase domains at the cytoplasmic side of the plasma membrane [17]. The receptor protein kinases (RPK) found in animal systems can autophosphorylate either on tyrosine residues or serine and/or threonine residues. Plant counterparts, receptor-like kinases (RLKs), have topological features of the tyrosine RPKs but contain sequence motifs characteristic of Ser/Thr kinases. In the past few years a number of RLKs have been cloned and characterized [43]. Although the kinase domains share a high degree of homology (about 40% identity at the amino acid level), their extracellular domains are very divergent. Based on the characteristics of the extracellular domains, several classes of RLK have been defined so far: the S-domain class, the leucine-rich repeat class, the epidermal growth factor (EGF)-like class (for review see [4]) and, more recently, the tumour necrosis factor receptor (TNFR)-like [2], the lectin-like [15] and thaumatin-like classes [44]. The S-domain class, which was the first to be identified, shows similarities to the *S*-locus glycoproteins involved in the self-incompatibility reaction in *Brassica* [31]. S-domain RLKs represent the largest family of transmembrane receptor kinases identified so far in plants [43]. The leucine-rich repeat class contains to date ten members (BRI1, TMK1, RLK5, TMLK1, PRK1, Xa21, LRK2, RPK1, CLAVATA and ERECTA) with differences in the number and organization of the repeats. The other classes are only represented by one member so far. *WAK1* from *Arabidopsis thaliana* [20] encodes a RLK

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers: AF027190, AF027191, AF027192, AF027193, AF027194, AF027195 and AF027196.

with an extracellular domain containing several EGF-like repeats. Herve *et al.* [15] isolated a new class of RLK by analysing two expressed sequence tags (EST) from *A. thaliana* showing homology to legume lectins. By screening an *A. thaliana* library for homologues of the *S*-locus receptor-like kinase (SRK) glycoproteins, Wang *et al.* [44] isolated a RLK (PR5K) with an extracellular domain related to pathogenesis-related (PR) proteins. Finally, Becraft *et al.* [2] isolated the *crinkly4* (*cr4*) gene encoding a receptor-like kinase with an extracellular domain resembling those of the mammalian tumour necrosis factor receptors. All these classes have been defined based on the homology of the extracellular domains to previously characterized proteins, although none has been shown to bind a ligand and function as a receptor. So far, plasma membrane localization has been demonstrated only for SRK proteins [7, 38] and for WAK1 which is tightly associated with the cell wall [14]. Xa21, PRK1, CRINKLY4, BRI1, CLAVATA and ERECTA are the only receptor-like kinases for which a function has been established experimentally. Indeed, *Xa21* is a resistance gene against a bacterial disease [36] and PRK1 is involved in the postmeiotic development of microspores [26]. Mutant analysis suggested that the *CLAVATA* and *ERECTA* genes of *A. thaliana* are involved in the coordination of cell growth in the floral and shoot apical meristems [6, 41]. Analysis of the maize *crinkly4* mutation suggested a role for CRINKLY4 in leaf epidermis and aleurone differentiation [2]. BRI1 was identified through a screening for brassinosteroid-response mutants of *A. thaliana* [27]. The mutant phenotypes and the characteristics of the protein suggested that BRI1 is involved in the brassinosteroid signal transduction cascade.

Recently, we isolated a gene (*Lrk10)* encoding a receptor-like kinase which is located at the leaf rust *Lr10* disease resistance locus of wheat [10]. LRK10 contains a new type of extracellular domain and is encoded by a member of a gene family in hexaploid wheat. Here, we investigated molecular and biochemical characteristics of the wheat *Lrk* gene family (*wlrk)* and the encoded proteins.

Material and methods

Plant material and genetic analysis

The chromosomal localization of the *wlrk* gene family was determined by Southern blot analysis of a set of aneuploid nulli-tetrasomic lines of cv. Chinese Spring [35]. Studies concerning the detection of other resistance genes were conducted on sets of near-isogenic lines (NILs) for powdery mildew and yellow rust resistance genes respectively. The NILs with powdery mildew resistance genes, developed by Dr L.W. Briggle [5], resulted from 8 backcrosses to the wheat variety Chancellor as recurrent parent and the *Pm1* donor lines Axminster, CI 13836, AsII and Norka, the *Pm2* donor lines Ulka and Idead 59B, the *Pm3* alleles donor lines Asosan (*Pm3a*), Chul (*Pm3b*), *Triticum sphaerococcum* (*Pm3b*), Sonora (*Pm3c*), Triticale (*Pm3c*) and Michigan Amber (*Pm3f*), the *Pm4a* donor lines Khapli and Yuma, and the *Pm5* donor line Hope. The NILs with yellow rust resistance genes (*Yr1*, *Yr5*, *Yr7*, *Yr8*, *Yr9*, *Yr10* and *Yr15*) were developed and kindly provided by Dr C.R. Wellings. They resulted from 6 backcrosses to the wheat variety Avocet as recurrent parent.

Mapping in wheat was performed on 126 F_2 individuals from a cross between the leaf rust-susceptible Swiss variety Frisal and the resistant parent Thatcher *Lr10* (R.L. 6004, kindly provided by Dr P. Dyck), as well as on 204 F_5 recombinant inbred lines derived from a cross between the Swiss winter wheat variety Forno (*Triticum aestivum*) and the Swiss winter spelt cultivar Oberkulmer (*Triticum spelta*). Mapping in barley was performed on a population of 71 F_1 anther-derived double-haploid individuals of an intraspecific cross between the cultivars Igri and Franka [12]. Mapping in rye was performed on an F_2 population of 154 individuals derived from a cross between the rye inbred lines P87 and P105 [21].

For plasma membrane preparation, the wheat variety Dragon was grown in a greenhouse under natural light for four weeks.

Linkage analysis

Linkage estimation was based on the maximum likelihood method using MAPMAKER [24]. The recombination fraction was transformed to centimorgans (cM) according to Kosambi [22].

PCR amplification, DNA cloning and sequence analysis

Amplification of the extracellular domain of the *wlrk* genes was performed by PCR between a sense oligonucleotide (5′GAAAG**ATG**AGTAAATTACTTG-3′) spanning the translation start site and an antisense oligonucleotide (5′-TGAGGGTCAGGCATGCAG-3′)

corresponding to the end of the extracellular domain of *Lrk10*. The reaction was performed on 20 ng of genomic DNA as previously described [10]. The amplified fragments of 900 bp were then cloned into pGEMT (Promega) and sequenced on the ALFexpress automated sequencer (Pharmacia, Switzerland). Sequence analysis and comparisons were performed by using PCGENE software (Intelligenetics, Belgium).

Southern and northern blot hybridizations

Southern blot and RNA blot analysis were performed as previously described [10].

Isolation of recombinant protein corresponding to the extracellular domain of LRK10 in Escherichia coli and production of polyclonal antibodies

The plasmid *pLRK10-A* containing the extracellular domain of *Lrk10* [10] was digested with *Sph*I/*Pst*I and the resulting fragment of 510 bp was cloned into the vector pQE30 (QIAexpressionist system, Qiagen, Switzerland) digested with the same enzymes. The construct was used to transform BL21 cells. The resulting plasmid *pLRK10ECD* was sequenced across the cloning sites to confirm the expected sequence. The recombinant protein starts with 6 histidine residues encoded by the vector followed by a sequence of 170 amino acids from the extracellular domain of LRK10. 250 ml 2× YT (50 μg/ml kanamycin, 100 μg/ml ampicillin) medium was inoculated with 3 ml of an overnight culture of BL21 cells harbouring *pLRK10ECD* and grown subsquently at 37 °C with shaking until the A_{600} reached 0.8. Isopropyl-β-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.5 mM to induce the production of recombinant protein. After 3 h of culture at 30 °C, the cells were harvested and the recombinant protein was purified under denaturing conditions as recommended by the manufacturer (Qiagen). Aliquots of each eluted fraction were run on a 12% acrylamide SDS-PAGE gel. Pure recombinant protein fractions in 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl pH 4.4, were dialysed overnight at 4 °C against 4 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl pH 7, then for 3 h against 2 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl pH 7. After a last dialysis against 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 7, the protein concentration was estimated [3]. 700 μg of pure recombinant protein was used to immunize two rabbits and to produce polyclonal antibodies (Readysystem, Switzerland).

Plasma membrane preparation and immunodetection of Lrk10 *antigens in wheat leaf extracts*

Leaves were excised at 6 °C and homogenized immediately in cold buffer containing 50 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.5, 5 mM EDTA, 330 mM sucrose, 2 g/l casein hydrolysate, 0.6% PVPP, 5 mM DTT, 1 mM PMSF, 10 mM ascorbate. Plasma membranes were purified from the microsomal fraction (10 000×–50 000 × *g* pellet), derived from 125 g of leaves, by partitioning in an aqueous polymer two-phase system [18, 25] composed of 6.5% Dextran T500 (Pharmacia, Sweden), 6.5% PEG 3350 (Union Carbide, USA) in 5 mM potassium phosphate buffer pH 7.5, 4 mM KCl. The hydrophobic plasma membrane proteins were then separated from the hydrophilic proteins by fractionation in 1% (w/v) Triton X-114, 150 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA [1, 19] as described by Stöhr *et al.* [39]. The final aqueous phase (hydrophilic proteins) and Triton phase (hydrophobic proteins) were then stored at −80 °C and used for electrophoretic studies.

SDS-PAGE [23] was performed with 8% acrylamide gels on a BioRad mini gel apparatus. The molecular weight markers corresponded to the broad-range SDS-PAGE standards from BioRad (USA). Proteins were electroblotted onto PVDF membranes (MSI, USA) as described by Towbin *et al.* [42]. Membrane blocking for 45 min and antibody (dilution 1/3000) binding overnight at 4 °C were carried out in Tris-buffered saline (TBS) pH 7.2 and 0.05% Tween 20 containing 2% (w/v) non-fat dry milk powder. After incubation with primary and secondary antibodies, blots were washed 3 times 10 min with TBS buffer, 0.05% Tween 20. Primary antibody binding was detected with alkaline phophatase-conjugated secondary antibody (IgG, Promega, USA).

Results

Lrk10 *belongs to a gene family which maps on group 1 chromosomes in hexaploid wheat and on homoeologous chromosomes in other cereal genomes*

The extracellular domain of *Lrk10* was used as a probe (*pLRK10-A*) on a Southern blot of DNA isolated from different wheat and spelt varieties (Frisal, Thatcher, Forno, Oberkulmer, Arina, Chinese Spring) and digested with seven restriction enzymes (*Eco*RI, *Bam*HI, *Eco*RV, *Hin*dIII, *Xba*I, *Bgl*II and *Dra*I). Six to ten fragments hybridized in each variety, demonstrating

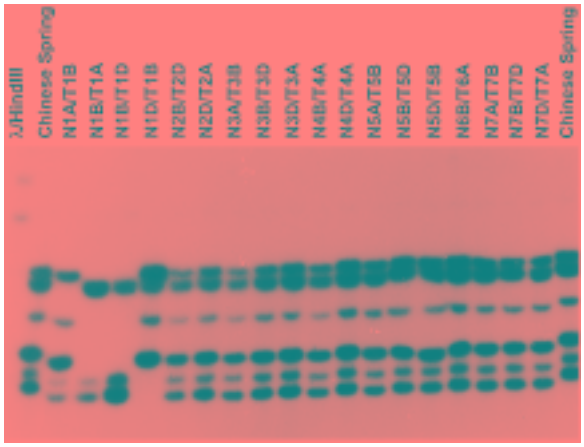

Figure 1. Southern blot hybridization of *Hin*dIII-digested genomic DNA of the wheat variety Chinese Spring and the derived aneuploid nulli-tetrasomic (NT) lines probed with *pLRK10-A*. Of the six fragments hybridizing in Chinese Spring one is missing in the N1A/T1B (nullisomic for chromosome 1A, tetrasomic for chromosome 1B) line, three are missing in the N1B/T1A, N1B/T1D lines and two in the N1D/T1B line, indicating their localization to the group 1 chromosomes.

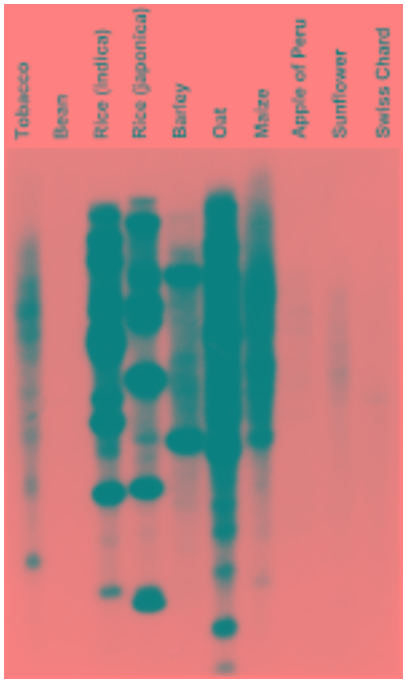

Figure 2. Southern blot hybridization of *Hin*dIII-digested genomic DNA extracted from different plant species probed with *pLRK10-A*. DNA was extracted from dicotyledonous (tobacco, bean, apple of Peru, sunflower and Swiss chard) and monocotyledonous (indica and japonica rice, barley, oat and maize) plant species. Hybridization were carried out under stringent conditions (60 °C).

the presence of a number of related genes in these genomes (data not shown). In order to localize the genes on the wheat chromosomes the *pLRK10-A* probe was hybridized with DNA isolated from a set of aneuploid nulli-tetrasomic lines of Chinese Spring. In these lines, the localization of a fragment is demonstrated by the absence of a hybridizing band as compared to Chinese Spring. Six bands were hybridizing in Chinese Spring and all of them were located on group 1 chromosomes (Figure 1). Indeed, one fragment of 3.8 kb corresponded to a fragment located on chromosome 1A, three bands of 4.5 kb, 3 kb and 2.3 kb were located on chromosome 1B whereas two fragments of 2 kb and 1.8 kb were on chromosome 1D.

To determine whether homologues of the *wlrk* gene family were present in other plant genomes we performed a Southern blot analysis with genomic DNA extracted from dicotyledonous (tobacco, bean, sunflower, apple of Peru and Swiss chard) and monocotyledonous (rice, barley, oat, maize) plant species. Under stringent hybridization conditions (60 °C), the *pLRK10-A* probe revealed very strong signals with the DNA of monocotyledonous plants whereas only a weak signal could be detected in tobacco and almost no signal in other dicot species (Figure 2). This result showed that the *wlrk* gene family is present with a high degree of homology at the sequence level in other cereal genomes. The complexity of the hybridization pattern varied from 2 bands in barley to 15 in oat, demonstrating a different organization of the loci homologous to *wlrk* in the different cereal genomes. We then mapped the *pLRK10-A* probe in Triticeae genomes of barley and rye. The results (Figure 3) demonstrate a good conservation of the *wlrk* locus at the end of the short arm of the homoeologous chromosome 1 in these species. Indeed, the *pLRK10-A* probe was mapped on top of chromosome 1RS in rye and 1HS in barley.

The pLRK10-A *extracellular domain probe reveals polymorphisms at several disease resistance loci in wheat*

We have previously shown that *Lrk10* maps to the *Lr10* leaf rust resistance locus in wheat [10]. In order to see whether other disease resistance loci might also encode receptor-like kinases similar to *Lrk10* Southern blots of near-isogenic lines (NILs) with powdery mildew (*Pm*) and yellow rust (*Yr*) resistance genes were hybridized with *pLRK10-A*. Polymorphisms were detected in *Yr* lines between the recurrent parent Avocet and two NILs containing the resis-

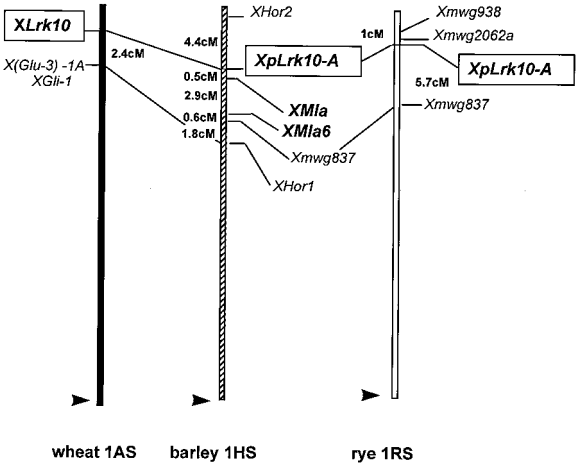

Figure 3. Mapping of the *pLRK10-A* probe in different cereal genomes. In barley and rye *pLRK10-A* maps to the distal end of the short arm of chromosome 1HS and 1RS respectively, very similar to the location on chromosome 1AS of hexaploid wheat.

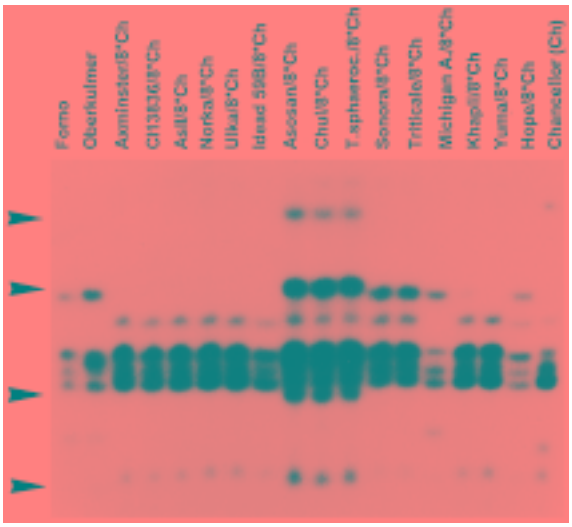

Figure 4. Southern blot hybridization of *Hin*dIII-digested DNA from NILs with powdery mildew resistance genes probed with *pLRK10-A*. The arrowheads indicate the four polymorphic bands observed in the line Asosan/8*Chul (*Pm3a*) and in the lines Chul/8*Ch and *T. sphaerococcum*/8*Ch which both contain the *Pm3b* resistance gene from different origins in the same genetic background.

tance genes *Yr1* and *Yr9* (data not shown). *Yr1* originates from the wheat gene pool and has been located on chromosome 2A whereas *Yr9* is located on the 1B/1R translocation [29]. With the powdery mildew NILs, we detected polymorphisms between the susceptible line Chancellor (Ch) and the near isogenic lines containing different alleles of the *Pm3* gene, Asosan/8*Ch (*Pm3a*), Chul/8*Ch (*Pm3b*), *Triticum sphaerococcum*/8*Ch(*Pm3b*), Sonora/8*Ch (*Pm3c*), Triticale/8*Ch (*Pm3c*) and Michigan Amber/8*Ch (*Pm3f*) (Figure 4). Polymorphisms were also detected with the NIL Hope/*8Ch containing the *Pm5* resistance gene (Figure 4). *Pm5* has been mapped on chromosome 7BL whereas *Pm3* has been mapped on chromosome 1AS in the same region as the *Lr10* resistance gene.

WLRK gene products show conserved regions within the extracellular domain

We designed two primers hybridizing to the 5′ and the 3′ end of the coding region for the extracellular domain of *Lrk10* to amplify by PCR representative members of the wheat *wlrk* gene family. We amplified and cloned products of 900 bp from genomic DNA of the spring wheat varieties Thatcher and Frisal. In these varieties, Southern hybridization with the extracellular domain of *Lrk10* as a probe revealed 6 and 9 hybridizing fragments, respectively. Comparison of the sequence of 8 cloned products (4 from Thatcher, 4 from Frisal) at the nucleotide and amino acid levels showed an overall homology of about 66% (Figure 5A). Alignment of the amino acid sequences showed distinct regions. A first conserved domain (C1) at the N-terminus which consisted of 58 amino acids including the signal peptide (Figure 5B) showed 97% homology among the different sequences. Within C1, a glycine residue located at position 18 was conserved in all the sequences and might correspond to a myristoylation site. The C1 region was followed by a variable region (V1) of 12 to 18 residues which contained either a block of serine or a block of alanine residues. Computer predictions of secondary structure of the V1 domain of LRK10 and FR36LRK (Figure 5A) showed that the serine residues were most likely in a β-turn conformation whereas the alanine residues were in an α-helix configuration. Two cysteine residues were remarkably conserved within the V1 variable region (Figure 5A). Two conserved domains (C2 and C3) showing 84% homology and two very variable regions V2 and V3 (Figure 5B) completed the extracellular domain. Among all the sequences 12 cysteines residues were totally conserved even when located in the more variable regions (Figure 5A). Interestingly, it has been demonstrated that a cluster of 12 invariant cysteines residues is a hallmark of the *S*-domain-containing genes from *Brassica* [30]. Several other features indicated some homology between the WLRK and the *S*-domain-containing gene products. We previously described a short sequence

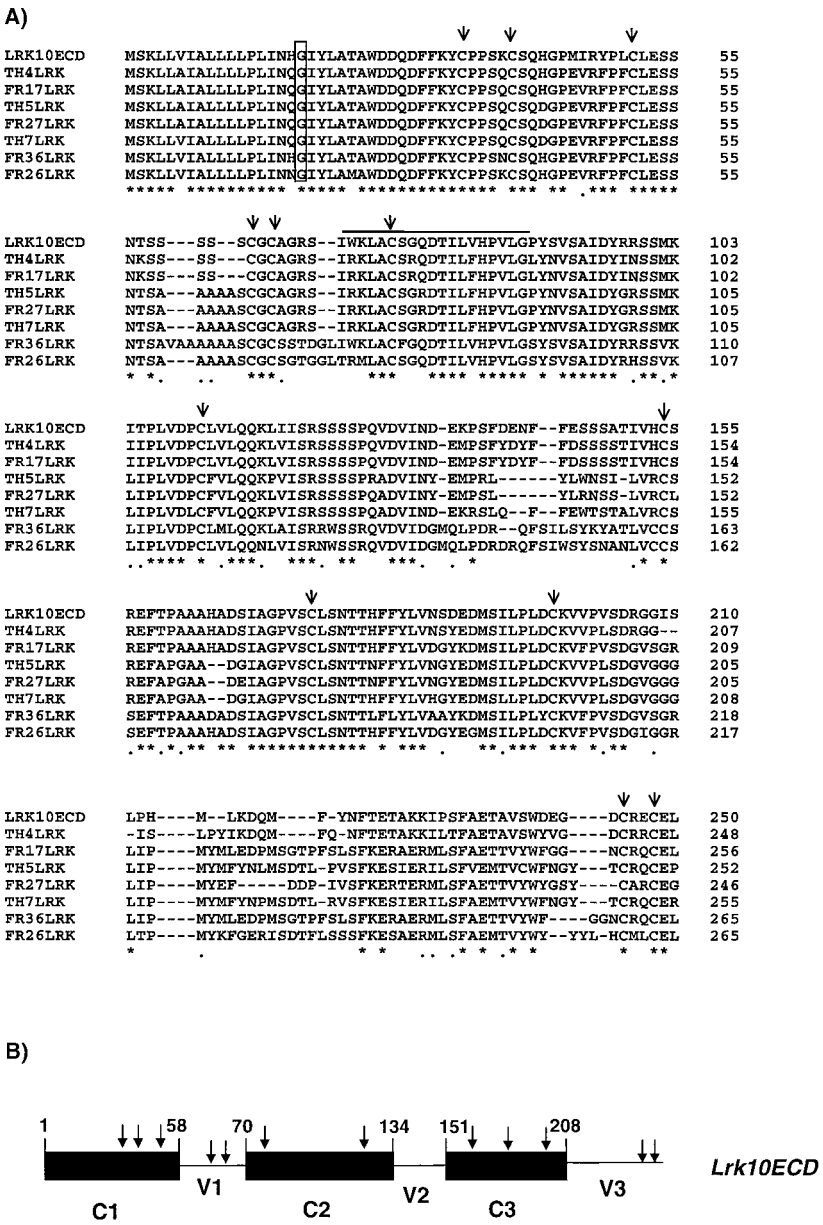

Figure 5. A. Amino acid sequence comparison of the extracellular domains of 8 different members of the *WLRK* gene family. Identical residues are indicated by an asterisk whereas similar residues are indicated by dots. The arrowheads indicate the 12 invariant cysteines. The upper bar delimits the sequence W-$(X)_7$-DT-X-L-$(X)_4$-LG showing homology with the SRK protein. The open box corresponds to a putative myristoylation site. B. Schematic representation of the 3 conserved domains (C1, C2, C3) and the 3 variable domains (V1, V2, V3) identified by comparison of the different *wlrk* gene family products. The position of the domains are numbered according to the *Lrk10* sequence.

of 6 residues W-$(X)_7$-DT-X-L-$(X)_4$-LG which were conserved between SRK and LRK10 [10]. The data obtained here confirmed this observation because of a total conservation of this motif except for the tryptophane residue which in some cases is replaced by an arginine (Figure 5A). In addition, two putative N-linked glycosylation sites with the consensus sequence N-X-S/T are conserved in positions corresponding to amino acid 56 and 184 of the LRK10 sequence. The first site is located after the first three invariant cysteines and before two cysteines only separated by one amino acid (C-X-C). This structure resembles the one

found in RLKs of *Brassica* and *Arabidopsis* having a *S*-domain [9, 37] (Figure 6).

Wlrk *genes are only expressed in aerial parts of the plant body*

We have previously shown that the *wlrk* gene family is constitutively expressed in seedling leaves [10]. To further study the spatial expression of these genes we performed a northern blot with 3 μg of mRNA isolated from wheat leaves, culms, spikelets and roots (Figure 7). Hybridization with the *pLRK10-A* probe corresponding to the extracellular domain of *Lrk10* revealed a stronger expression of a message of 2.1 kb in the leaves of seedlings and flag leaves than in the culm and the spikelets. No expression was detected in the roots. This result indicated that the *wlrk* gene family is specifically expressed in the aerial parts of the plant.

WLRK proteins are present in the plasma membranes

Based on the deduced amino acid sequences, *wlrk* genes were expected to encode plasma membrane receptors having an extracellular domain which is separated from the kinase domain by a transmembrane sequence. To test this hypothesis polyclonal antibodies against the extracellular domain of LRK10 were raised in rabbits. The subcellular locations of WLRK proteins were determined by protein immunoblots following SDS-PAGE separation of polypeptides present in different subcellular fractions (Figure 8). A 70 kDa polypeptide was detected in the whole homogenate of wheat leaves but not in the soluble fraction (Figure 8, lane 1 and 2). This polypeptide was also not detected with pre-immune serum (data not shown). In addition, the 70 kDa protein was highly enriched in the microsomal fraction containing membrane vesicles originating from all intracellular membranes as well as from the plasma membrane (Figure 8, lane 3), and showed an enrichment in the plasma membrane fraction as compared to the intracellular membrane fraction (Figure 8, lane 5 and 4). The band seen at 70 kDa in the intracellular membrane fraction (lane 4) could be due to inside-out plasma membrane vesicles partitioning to the bottom phase whereas right side-out plasma membrane vesicles partition to the top phase (lane 5) in the two-phase system used [18]. When using Triton X-114 fractionation to separate intrinsic (hydrophobic, integral plasma membrane proteins) from extrinsic (peripheral, water soluble plasma membrane proteins), the 70 kDa species was detected only in the intrinsic plasma membrane protein fraction (Figure 8, lanes 6 and 7). In addition to the 70 kDa species, the antiserum recognized three intrinsic plasma membrane proteins which could correspond to other members of the WLRK protein family (Figure 8, lane 6). Several extrinsic plasma membrane polypeptides (Figure 8, lane 7) were recognized by the antiserum, suggesting that these extrinsic proteins share antigenic epitopes with the extracellular domain of the LRK10 protein. The 70 kDa protein recognized by the ECD10 antiserum has the expected size of the LRK10 protein (71 kDa) and is an intrinsic plasma membrane protein.

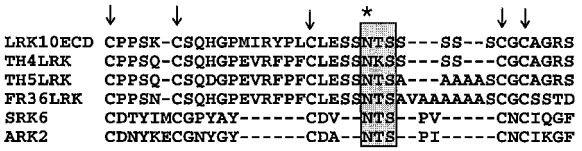

Figure 6. Comparison of amino acid sequences encoded by the *wlrk* gene family from wheat and two *S*-domain containing genes from *Brassica* [9, 37]. The arrowheads indicate the cysteine residues conserved in both families. The boxed amino acid sequence NTS corresponds to a putative N-glycosylation site with the N indicated by an asterisk.

Discussion

The WLRK extracellular domain: features and similarities with S*-domain receptor-like kinases*

RLK proteins having an extracellular domain of the LRK10 type are encoded by a gene family in wheat. The number of *wlrk* genes was estimated by Southern hybridization to be between 6 and 10 in the different wheat varieties analysed. The comparison of the extracellular domain at the amino acid level of 8 different members of the *wlrk* gene family revealed 3 very conserved and 3 more variable regions. These features characterize a new type of extracellular domain. Interestingly, some of the conserved residues showed a similar organization as found in proteins having *S*-domains and which are involved in self incompatibility in some species. Indeed, an identical number (12) of cysteine residues is conserved in both WLRK, SRK (*S*-locus receptor-like kinase) and SLG (*S*-locus glycoprotein) proteins. The position of these residues in the extracellular domain is not identical but one cannot exclude the possibility of a common type of secondary structure. In addition, in a short region encompassing a conserved N-glycosylation site of the WLRK and SRK gene products comparable distances between the cysteine residues were found.

These similarities between the *wlrk* and the *srk* genes agree with our previous suggestion [10] that the two types of genes might have a common origin. We have previously shown that LRK10 is encoded at the leaf rust resistance locus *Lr10* in wheat. Recently, we have found that *Lrk10* is located in a gene-rich region (C. Feuillet, unpublished data). Interestingly, molecular analysis of plant disease resistance loci in plants have shown the presence of gene clustering at these loci [40]. Together with a primary structure resembling a receptor-like kinase, this makes LRK10 a candidate for being involved in the signal transduction after pathogen infection. Similarities between self-incompatibility and host-pathogen interactions have recently been discussed [8] and from a morphological point of view, this is particularly evident for a fungal pathogen. There is emergence of an elongated cell (germ tube or pollen tube) from a spore-like structure (fungal spore or pollen grain) on the surface of the host and growth of the tube within or between the cell walls [32]. Both *Xa21* and *PR5K* are encoding receptor-like kinases and have been implicated in plant defense responses: XA21 was identified as a resistance gene product and PR5K has an extracellular domain related to PR proteins. Recently, evidence for a role of an *S*-domain containing receptor-like kinase in plant defence was reported. Pastuglia *et al.* [32] demonstrated a transient induction of the *SFR2* gene upon infection and wounding with a kinetic and induction pattern typical of defence genes. These observations raised the possibility of a common ancestor for genes involved in self-incompatibility and defence. Furthermore, Clark *et al.* [6] suggested a common origin for disease resistance and developmental signalling pathways based on similarities of the gene structure of receptor-like kinase genes such as *CLAVATA1* and *Xa21*. It is interesting to speculate that different types of RLKs involved in different signalling pathways might have a common origin.

Wlrk *genes map to homoeologous chromosomes in Triticeae and to several disease resistance loci in wheat*

Southern hybridization with the extracellular domain of *Lrk10* (*pLRK10-A*) revealed that all the hybridizing fragments were specifically located on the group 1 chromosomes in wheat. Wheat is hexaploid and contains 3 genomes (A, B, D). Thus, the specific location of different genes probably reflects orthologous loci on 1A, 1B and 1DS, respectively. Comparative mapping on diploid cereal species, such as barley and rye,

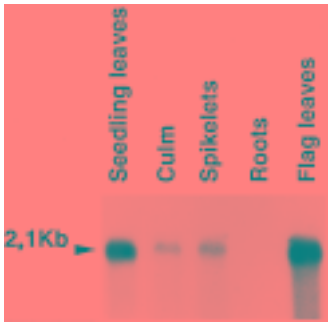

Figure 7. Northern blot hybridization of poly(A)$^+$ RNA isolated from different wheat organs and probed with *pLRK10-A*. The 2.1 kb mRNA expressed in the aerial parts of the plant is indicated by an arrowhead.

demonstrated a similar localization of the *wlrk* gene homologues in these species. Indeed, *pLRK10-A* was mapped on the top of chromosome 1HS in barley and 1RS in rye. These results indicate a clear conservation at this locus among the Triticeae. Good collinearity between A, B, D, H and R genomes has already been shown, for instance for powdery mildew resistance loci [13, 34]. Here, collinearity was also found with genes surrounding the *wlrk* loci in wheat and barley. In barley, *pLRK10-A* was mapped at 4.4 cM from two high-molecular-weight (HMW) seed storage protein loci (Hordein 2 and Hordein 1). In wheat, we previously mapped *Lrk10* in two mapping populations at 8 cM [33] and 2.4 cM [11] respectively, from *GluA-3*, a gene encoding a low-molecular-weight (LMW) seed storage protein (glutenin).

Near-isogenic lines (NILs) with resistance genes against yellow rust and powdery mildew revealed polymorphisms with the probe *pLRK10-A* suggesting that disease resistance loci other than *Lr10* encode WLRK homologues. In the yellow rust NILs, lines with the *Yr1* and *Yr9* resistance genes showed polymophisms if compared with the recurrent parent Avocet. *Yr9* is located on the 1B/1RS translocation which contains several resistance genes such as *Lr26* and *Sr31* [29]. In the case of a wheat/rye translocation, it is not surprising to find polymorphisms. On the contrary, *Yr1* originated from the wheat gene pool and therefore represents a good candidate for further analysis of the linkage between the yellow rust resistance and the polymorphic band. In NILs with powdery mildew resistance genes, polymorphisms were detected between the recurrent parent Chancellor and the NILs containing either different alleles of the *Pm3* gene or the *Pm5* gene. The *Pm3b* gene was introduced into the NILs from two independent sources, Chul

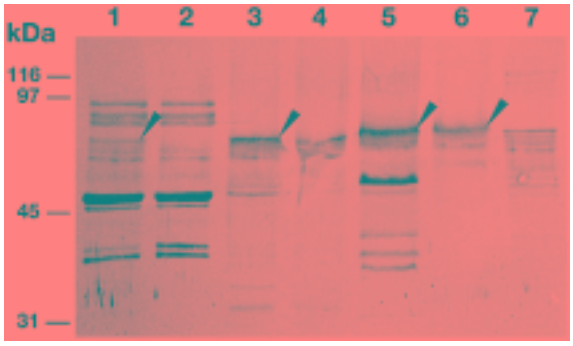

Figure 8. Association of the LRK10 protein with the plasma membrane in wheat leaves. Subcellular fractions were obtained by differential centrifugation and aqueous polymer two-phase partitioning. The proteins were resolved by SDS-PAGE, electroblotted and the blots subsequently probed with an antiserum raised against the extracellular domain of LRK10. Among the proteins recognized by the antiserum, a polypeptide of about 70 kDa (arrowhead) was present in the whole homogenate (lane 1), was not present in the soluble fraction (lane 2), and was enriched in the microsomal membrane fraction (lane 3). This polypeptide primarily partitioned to the plasma membrane fraction (lane 5), as compared to the intracellular membrane fraction (lane 4). Fractions obtained by Triton X-114 fractionation suggest that the 70 kDa protein has the properties of an intrinsic, hydrophobic plasma membrane protein (lane 6) as compared to water-soluble, extrinsic plasma membrane proteins (lane 7). The arrowheads indicate the 70 kDa polypeptide recognized by the antiserum.

and *Triticum sphaerococcum*, which are land races from different geographical areas [28]. Interestingly, both NILs showed the same polymorphic bands when hybridized with the extracellular domain of *Lrk10*. The *Pm3* gene was mapped through classical genetic analysis on chromosome 1AS in the same region as *Lr10*. It mapped at 1–5 cM from the Hairy glumes (*Hg*) gene whereas *Lr10* was mapped at 6 cM from the same gene [29]. We are currently analysing an F_2 population from a cross between Chul/8*Chancellor × Chancellor in order to study the linkage between the resistance gene and the polymorphic bands.

Wlrk *genes are specifically expressed in the aerial parts of the plant and encode receptor-like kinases which are located in the plasma membrane*

The *wlrk* gene family in wheat was found to be specifically expressed in the aerial parts of the plant. The expression was stronger in the seedling and adult leaves than in the culms and spikelets. No expression was detected in the roots. This supports the idea that the *wlrk* gene family encodes receptors which might be involved in the recognition of a pathogen (*Puccinia recondita* f. sp. *tritici*) developing in the aerial parts of the plant and infecting the leaves. The different types of plant RLKs isolated so far show various patterns of expression: while some are constitutively expressed in specific reproductive or vegetative tissues, others are developmentally regulated in both types of tissues. Only one member of the *S*-gene family (SFR2) has been shown to be induced by a range of stimuli inducing plant defence [32] whereas RPK1 from *A. thaliana* has recently been shown to be induced by osmotic stress [16].

Members of the *S*-locus protein family and the EGF class of receptor-like kinases are the only RLK proteins which have been shown to be localized to the plasma membrane whereas the subcellular localization of the others is unknown. Delorme *et al.* [7] and Stein *et al.* [38] demonstrated a plasma membrane localization of SRK proteins from different haplotypes. WAK1, belonging to the epidermal growth factor (EGF) class of RLKs, was also found to be associated with the plasma membrane. Moreover, WAK1 appeared to be tightly associated with the cell wall and might be involved in a physical connection between the extracellular matrix and the cytoplasm [14]. Here, we found that antibodies against the extracellular domain of LRK10 reacted with a protein of the expected size (70 kDa) that was present in plasma membranes of leaf cells. This protein, which was not detected with the preimmune serum, was absent in the soluble fraction. In addition to plasma membranes, the 70 kDa protein was also found to a lesser extent in the endomembrane fraction. These data support the hypothesis of WLRK proteins being plasma membrane receptors. Fractionation in Triton X-114 showed that the 70 kDa protein is an intrinsic membrane protein confirming the suggestion that LRK10 and the other WLRK proteins are transmembrane receptors [10]. The immunoreactive protein found in the endomembrane fraction possibly corresponds to precursors of the plasma membrane-associated protein being processed through the endoplasmic reticulum and Golgi compartments. Indeed, WLRK proteins contain a putative signal peptide as well as N-glycosylation sites. Apart from recognizing a 70 kDa polypeptide, the ECD10 antiserum labelled three intrinsic plasma membrane proteins that could represent other members of the WLRK protein family sharing common antigenic epitopes in the three very conserved regions of the extracellular domains. The extrinsic plasma membrane proteins recognized by the antiserum are either more hydrophilic forms of WLRK proteins, possibly due to extensive glycosylation at the con-

served N-glycosylation site, or may represent other proteins of the plasma membrane possessing similar or identical antigenic epitopes as present in the three conserved regions of the extracellular domain of the LRK10 protein. That more than one polypeptide is immunologically recognized was expected since the serum was raised against a protein domain with several amino acid stretches highly conserved in the WLRK family of proteins. However, only one of the polypeptides recognized has the molecular weight expected from the coding region of the *Lrk10* gene. Furthermore, this polypeptide behaves, in the Triton X-114 fractionation, as an intrinsic plasma membrane protein as would be expected from the deduced amino acid sequence of the *Lrk10* gene.

We conclude that molecular and biochemical data are in agreement with the hypothesis that WLRK proteins are receptors located in the plasma membrane. Furthermore, genetic analysis suggested that *wlrk* genes are present at different disease resistance loci. These results raise the question whether WLRK proteins are directly involved in the resistance reaction against fungal pathogens in wheat. Additional genetic analysis and transformation experiments should allow a better understanding of the function of WLRK proteins as well as about the relationship between *wlrk* and disease resistance genes in wheat.

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